수의학석사학위논문

Cloning of the Gyeong-ju Donggyeong dog using trypsin-treated cells during the primary culture

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2017 년 8 월

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이 논문을 수의학 석사학위논문으로 제출함

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Cloning of the Gyeong-ju Donggyeong dog using trypsin-treated cells during the primary culture

by Yoo Bin Choi

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF THE MASTER OF SCIENCE

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Department of Veterinary Medicine, Graduate School

Seoul National University

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Cloning of the Gyeong-ju Donggyeong dog using trypsin-treated cells during the primary culture

Yoo Bin Choi

(Supervisor: Byeong Chun Lee, D.V.M., Ph.D.)

Veterinary Clinical Sciences

Department of Veterinary Medicine, Graduate School

Seoul National University

ABSTRACT

Somatic cell nuclear transfer (SCNT) has been considered as a useful tool to maintain genetic information of animals and it has demonstrated their potential application for conserving endangered animals. Gyeong-ju Donggyeong dog, a

natural monument in Korea since 2012, has the oldest history among the Korea natural monument breeds. However, the number of this genetically valuable breed had been reduced gradually from period of Japanese occupation. Recently, it is reported that only about four hundred individuals is remained in Gyeong-ju and this breed is classified as endangered. Therefore, it is essential to save this natural monument breed from extinction and maintain a pure descent.

Primary culture is the most basic and essential procedure to establish donor cells needed for SCNT. However, in case of endangered animals there is a difficulty about obtaining tissues, which must be required for primary culture. Thus, it is necessary to construct the primary culture method which can secure a large number of somatic cells with the given quantity of tissue. Trypsin has been widely used in primary cultures to rapidly harvest large numbers of cells. However, there are few studies on the effects of trypsin treatment on primary culture and canine SCNT efficiency using trypsin-treated cells.

To investigate the effect of trypsin treatment on primary culture, I compared harvested cell numbers, cell viability, transcript levels of cell apoptosis-related and growth-related genes and *in vivo* embryo development after SCNT between a control (trypsin non-treated) group and a trypsin-treated group. To establish the trypsin-treated donor cell line (trypsin-treated group), Minced skin tissue was treated with 0.25% (w/v) trypsin ethylenediamine tetra-acetic acid (EDTA) at 37 ℃ for 1 h before seeded and another was non-treated (control group).

As a result, compared to the control group trypsin treatment in primary culture had a trend ($P = 0.14$) to isolate more numbers of somatic cells in the same period. However, there was no significant difference in cell viability and expression of

Bax/Bcl2 ratios between control and trypsin treated groups. In regard to transcript levels of cell growth-related genes, the trypsin-treated group contained significantly higher *PHB* transcript levels, but it showed significantly lower *Akt1* transcript levels compared to the control group. Among the three pairs (each consisting of control and trypsin treated groups) of cell lines, which were used in the above experiments, one pair of the control and trypsin treated cell line was used for SCNT to compare the cloning efficiency. A total of 90 of cloned embryos derived from the control group were transferred to six recipients and 71 cloned embryos derived from trypsin-treated group were transferred to five recipient dogs. In each group, three surrogate mothers became pregnant (control group, 50.0%; trypsin-treated group, 60.0%) and delivered four pups (control group, 4.4%; trypsin-treated group, 5.6%).

Based on these results, trypsin was treated during the primary culture procedure to establish a Donggyeong dog cell line. The Donggyeong dog cells-were used for SCNT and a total 42 cloned embryos were transferred to three recipient dogs. One surrogate mother got pregnant (33.3%) and one cloned Donggyeong dog was delivered by caesarean section (2.3%). The cloned Donggyeong dog was genetically identical with the donor Donggyeong dog and also had a short-tail which is one of the specific characteristics of Donggyeong dogs.

In conclusion, trypsin treatment in primary culture did not affect cell viability, expression of *Bax/Bcl2* ratio and canine cloning efficiency and this method tended ($P = 0.14$) to increase the cell number compared to the control method. The present study demonstrated for the first time the successful production of cloned Gyeong-ju Donggyeong dog by nuclear transfer using donor cells derived from trypsin-treated

primary culture method. In addition, I demonstrated that the unique phenotype of the Donggyeong dog could be conserved by SCNT.

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Key words: somatic cell nuclear transfer, Gyeong-ju Donggyeong dog, trypsin, primary culture

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LIST OF ABBREVIATIONS

bp	**Base pair**
BSA	**Bovine serum albumin**
cDNA	**Complementary DNA**
DMEM	**Dulbecco's modified eagle's medium**
DPBS	**Dulbecco's phosphate buffered saline**
DMSO	**Dimethyl sulfoxide**
DNA	**Deoxyribonucleic acid**
ET	**Embryo transfer**
EDTA	**Ethylenediamine tetra-acetic acid**
FBS	**Fetal bovine serum**
mtDNA	**Mitochondrial DNA**
PCR	**Polymerase chain reaction**
PVS	**Perivitelline space**
P4	**Progesterone**
RT	**Reverse transcript**
SCNT	**Somatic cell nuclear transfer**

PUBLICATION LISTS

PUBLICATION PAPERS

1. **Choi, Y. B.**, Kim, G. A., Oh, H. J., Kim, M. J., Jo, Y. K., Setyawan E. M., Lee, S. H., Lee, B. C. Cloning of the short-tailed Gyeong-ju Donggyeong dog *via* SCNT: conserving phenotypic inheritance. J Vet Med Sci. 2016; 78:329-31.
2. Saadeldin, I. M., Kim, S. J., **Choi, Y. B.**, Lee, B. C. Post-maturation zona perforation improves porcine parthenogenetic trophoblast culture. Placenta. 2014; 35:286-8.
3. Saadeldin, I. M., Kim, S. J., **Choi, Y. B.**, Lee, B. C. Improvement of cloned embryos development by co-culturing with parthenotes: a possible role of exosomes/microvesicles for embryos paracrine communication. Cell Reprogram. 2014; 16:223-34.
4. Kim, M. J., Oh, H. J., Kim, G. A., Suh, H. N., Jo, Y. K., **Choi, Y. B.**, Kim, D. H., Han, H. J., Lee, B. C. Altering histone acetylation status in donor cells with suberoylanilide hydroxamic acid does not affect dog cloning efficiency. Theriogenology. 2015; 84:1256-61.
5. Setyawan, E. M., Kim, M. J., Oh, H. J., Kim, G. A., Jo, Y. K., Lee, S. H., **Choi, Y. B.**, Lee, B. C. Maintaining canine sperm function and osmolyte content with multistep freezing protocol and different cryoprotective agents. Cryobiology. 2015; 71: 344-9.

6. Oh, H. J., Choi, J., Kim, M. J., Kim, G. A., Jo, Y. K., **Choi, Y. B.**, and Lee, B. C. Propagation of elite rescue dogs by somatic cell nuclear transfer. Anim Sci J. 2016; 87: 21-6.
7. Setyawan, E. M., Kim, M. J., Oh, H. J., Kim, G. A., Jo, Y. K., Lee, S. H., **Choi, Y. B.**, Lee, B. C. Spermine reduces reactive oxygen species levels and decreases cryocapacitation in canine sperm cryopreservation. Biochem Biophys Res Commun. 2016; 479: 927-32.

PART I

GENERAL

INTRODUCTION

1. Literature review

1.1. Gyeong-ju Donggyeong dog

In 2012 the Gyeong-ju Donggyeong dog was listed on Korean Canine Natural Monument (Cultural Heritage Administration of Korea, number: 540). The name of Gyeong-ju Donggyeong dog originated from the capital of ancient Silla kingdom in Korea and it has been found in a Korean dictionary published in 1982, where the Donggyeong is defined as "a dog that used to be common in the ancient Gyeong-ju area". This Donggyeong dog has the oldest history among the Korean natural monument breeds. As an example, there are some Donggyeong dog shaped earthenware discovered, which made by citizens of the Silla Dynasty (B.C. 57-935). Also the oldest reference to the Donggyeong dog is in "The Chronicles of the Three Kingdoms", the book about Korea's Three Kingdoms era, the period between the fourth and seventh centuries, which was written during the Goryeo dynasty. In addition, Donggyeong dog is also referred to in many historic documents, such as Dongkyung jabki (published in AD 1669) and Sungho sasul (published in AD 1740) [1].

However, Gyeong-ju Donggyeong dogs were slaughtered during the period of Japanese occupation in Korea because Donggyeong dogs look similar with 'Komainu' which is the symbol of the auspicious dog in Japan. Even after that the Donggyeong dogs were killed due to the shape of the tail, which was considered 'abnormal' or 'unfortunate'. In the end, the population of Donggyeong dogs had been on the decline and classified as endangered. In 2005, Gyeong-ju and

“Preservation Association for Gyeongju Donggyeong Dog” establish a dog breeding facility to protect the Donggyeong dog and designate it as a natural monument. “Korean Gyeong-ju Donggyeong Dog Association” was established in 2009 and since then, it has carried out the Donggyeong dog maintenance project such as studies on the body standardization. In 2010, Donggyeong dog got registered as a Koran Dog No. 4 by Korea Kennel Club, following Jindo, Poongsan and Sapsaree. Finally in 2012, Donggyeong dog was listed on Korean Natural Monument No. 540. Despite these efforts to preserve the dog they are still classified endangered and there are some 406 Donggyeong dog remaining in Gyeong-ju [1-3].

In terms of the appearance of Donggyeong dog, they are classified four color types (black, white, yellow and brindle) and looks very similar to the Jindo. However, the most distinctive features of Donggyeong dogs are at shape of the tail: While the Jindo’s tail is long and curved, but the Donggyeong dog has a short-tailed or tailless. According to the information from the “Korean Gyeong-ju Donggyeong Dog Association”, the short-tailed Donggyeong dog over twelve months old has a tail of around 11.38 ± 2.44 cm in length and five to nine of coccygeal vertebrae in radiographic observations. The tailless Donggyeong dog has one to four of coccygeal vertebrae, and adult dogs (over twelve months old) have a tail of around 6.3 ± 2.81 cm in length [1-3].

Even now there is much effort in “Korean Gyeong-ju Donggyeong Dog Association” to preserve Gyeong-ju Donggyeong dog and it is essential to find various methods for saving this natural monument breed from extinction and maintaining a pure descent.

1.2. Primary culture

Primary culture is that stage of the isolation of the cells from a piece of tissue or from tissue that is disaggregated by enzymatic or mechanical methods. In primary culture, there are four stages are included: (1) acquirement of the sample, (2) isolation of tissue, (3) dissection and/or disaggregation and (4) seeding tissue into the culture vessel [4]. Among these primary culture stages, several techniques (primary explant technique and disaggregation techniques) have been used for dissection and/or disaggregation stage. The primary explant technique was the original method developed by Harrion [5] and Carrel [6] to initiate a tissue culture. Briefly, tissue was fixed in blood plasma, mixed with heterologous serum and embryo extract and this supplement stimulated cell migration. This method is still used but these days it has been replaced by simplified methods using growth media instead of blood plasma etc. This technique is useful for small amounts of tissue due to the risk of losing cells in enzymatic and mechanical disaggregation method but it has disadvantage of poor tissue adhesiveness [7].

Enzymatic and mechanical disaggregation method are included in disaggregation techniques and these techniques can yield a higher number of cells in a shorter time. The most widely used enzymes for tissue disaggregation are trypsin, collagenase, pronase and Dispase. Trypsin [8, 9] and pronase [10, 11] can give the most complete tissue disaggregation but may damage the cells. Collagenase [12, 13] and Dispase [14, 15] give less damage but give incomplete tissue disaggregation [4].

Mechanical disaggregation method is chosen when there is a risk of proteolytic damage to cells. In mechanical disaggregation spillage, sieving and syringing method are included: Spillage method [16] is collecting the cells spilling

out when the tissue is sliced; sieving method is pressing the tissue up to the mesh to reduce the size of tissue; syringing method [17] is pipetting the tissue to minimize the tissue. The mechanical disaggregation method can give a cell suspension more quickly than enzymatic disaggregation method but it can cause the mechanical damage, and to obtain the cells successfully, it is recommended to use mechanical methods for soft tissue like a brain [4]

1.2.1. Trypsin

Trypsin is a member of the serine protease from the PA clan superfamily and found in a digestive system [18]. It was first named in 1876 by Kuhne who investigated the proteolytic activity of this pancreatic enzyme. Nowadays, trypsin continues to be used in the development of tissue and cell culture protocols [19-21]. Trypsin is the most widely used enzyme in the cell culture and it can decompose the cell surface protein and it can detach the cells both from cell culture dishes and from each other [4]. Also trypsin can endure well regardless of types of cells and its enzymatic activity can be easily neutralized by serum in cell culture medium. In addition, trypsin has been widely used as common enzyme used for tissue disaggregation in primary culture [7] due to its accessibility and easy of neutralization of the enzymatic activity.

1.3. Canine somatic cell nuclear transfer

Since the birth of "Snuppy" [22], the first cloned dog, canine SCNT has been considered as a useful tool to maintain genetic information of dogs. Canine SCNT has been applied for various objectives, such as cloning of pet dogs [23, 24], service dogs [25, 26] for preservation of their abilities, endangered species [27-29] for conservation them from extinction, and transgenic dogs [30, 31] for human diseases models.

1.3.1. Conservation of endangered canids using SCNT

Since many species are in danger of exertion (http://www.iucnredlist.org/), conserving the endangered animal is a big issue to many social organization in the world. To preserve the endangered animals, reproduction technologies have demonstrated their potential application [32-34]. Among the various reproductive technologies, SCNT is useful because it could be applied to maintain genetic information of endangered animals.

In case of endangered canids, the female gray wolf (Canis lupus) was cloned in 2005 using interspecies SCNT (iSCNT) [27]. There are some problems in cloning endangered animals such as collecting oocytes, preparing and caring recipients. For this reason, iSCNT has been used since it has potential advantages to obtain con-specific oocytes. To clone the gray wolf, dog oocyte were used for SCNT and cloned embryos were transferred to dog recipients. Cloned gray wolves were genetically identical to the donor wolf and the mtDNA was identical to that of the oocyte donor. Furthermore, in 2008 there was cloning attempt using fibroblast cells derived from

a male gray wolf 6 h after its death and three cloned wolf pups were delivered by interspecies SCNT [28].

In 2009, Sapsaree, which is registered as a Korean Natural Monument, was cloned by SCNT using mixed breed dog oocytes, to protect it from extinction and maintain a pure pedigree [29]. The clones were genetically identical to the somatic cell donor Sapsaree and mtDNA was identical to that of the oocyte donor. It has also proven that the cloned Sapsaree had similar hair coats with the somatic cell donor dog. From these studies, SCNT in canids could not only be used for preservation of endangered canine species involving sudden death but also for the conservation of phenotypic feature of a canine species.

2. General objective

The purpose of this study is to produce Gyeong-ju Donggyeong dog by SCNT using trypsin-treated cells during the primary culture. This thesis is composed of 5 parts. In part I, as a general introduction, it was explained the literature background about Gyeong-ju Donggyeong dog, application of SCNT for endangered species and primary culture. In part II, I described general methodology of this study. Part III showed the effect of trypsin treatment on primary culture and SCNT efficiency using trypsin-treated cells. Finally, Gyeong-ju Donggyeong dog was produced by SCNT using donor cells derived from trypsin-treated primary cells in part IV. In part V, a final conclusion of this study was described.

PART II

GENERAL

METHODOLOGY

1. Chemicals and materials

All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise stated.

2. Care and use of animals

Mixed-breed female dogs (Canis familiaris) between 1 and 5 years of age and weighing 20 to 35 kg were used as oocyte donors and embryo transfer recipients. The study was conducted in accordance with recommendations described in ‘‘The Guide for the Care and Use of Laboratory Animals’’ published by published by Seoul National University. Facilities for dog care and managed following a standard procedure established by the Committee for Accreditation of Laboratory Animal Care at Seoul National University.

3. Determination of ovulation and recovery of *in vivo* matured oocytes

In vivo matured oocytes were recovered approximately 72 h after ovulation [22]. In order to determine the ovulation day, blood samples (3-5 ml) of estrus bitches were collected and serum progesterone concentrations were analyzed by Immulite 1000 (Siemens Medical Solutions Diagnostics, Los Angeles, CA). The day of ovulation was considered the day that serum progesterone concentration reached 4.0-7.5 ng/ml. The dogs were first anesthesia with 4.0mg/kg of ketamine (Yuhan, Seoul, Korea) and 0.9 mg/kg of xylazine (Rompun, Bayer Korea, Seoul, Korea) and then anesthesia was maintained with isoflurance (Hana Pharm. Co., Ltd., Seoul, Korea).

After pulling the ovary, the fimbriated end of the oviduct was accessed through the bursal slit and cannulated using a flushing needle. An intravenous catheter (24 gauge) was inserted into the caudal portion of the oviduct, and the oviduct was flushed with 10 ml of medium (Hepes-buffered TCM-199 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% Fetal bovine serum (FBS, Invitrogen), 2 mM NaHCO3 and 5 mg/ml bovine serum albumin (BSA, Invitrogen)). Ovulated oocytes were retrieved within the flushed medium through flushing needle.

4. Somatic cell nuclear transfer

Collected *in vivo* matured oocytes were transported to the laboratory within 10 min in Hepes-buffered TCM-199 at 38.5 ℃, and cumulus cells were removed from the *in vivo* oocytes by repeated pipetting in 0.1% (v/v) hyaluronidase. Denuded oocytes were enucleated in a drop consisting of medium (Hepes-buffered TCM-199 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% FBS, 2 mM NaHCO3 and 5 mg/ml bovine serum albumin (BSA, Invitrogen)) supplemented with 5 μg/ml cytochalasin B and 5 μg/ml bisbenzimide (Hoechst 33342). A donor cell was transferred into the perivitelline space (PVS) of the enucleated oocytes. Couplets were then placed in a solution of 0.26 M mannitol, 0.1 mM MgSO4, 0.5 mM Hepes and 0.05% (w/v) BSA and fused with two pulses of direct current of 72 V for 15 μsec using an Electro-Cell Fusion apparatus (NEPA GENE Co., Chiba, Japan). After fusion, the fused couplets were chemically activated by a 4 min incubation with 10 μM calcium ionophore, followed by 2 h of culture in 1.9 mM 6-dimethylaminopurin [22, 35].

5. Embryo transfer and pregnancy diagnosis

Reconstructed embryos were surgically transferred into the oviducts of naturally synchronized recipient dog [22]. The oviduct of a recipient dog was exposed in the same method as described for the recovery of oocytes. Reconstructed embryos were placed in the ampullary portion of the oviduct using a 3.5-Fr Tom Cat Catheter (Sherwood, St. Louis, MO, USA). Pregnancies were detected around 26 days after embryo transfer using a SONOACE 9900 (Medison Co. LTD, Seoul, Korea) ultrasound scanner. All pregnant surrogates performed delivery either naturally or by caesarean section.

PART III

EFFECT OF TRYPSIN TREATMENT ON PRIMARY CULTURE OF CELLS FOR CLONING DOGS

1. Introduction

Since production of first cloned dog, Snuppy [22], the canine somatic cell nuclear transfer (SCNT) has provided a tool for propagating various canids [26, 27, 29, 31]. Still, producing cloned dogs by SCNT has been still relatively inefficient compared to other cloned species [36-38]. The successful cloning of dogs is influenced by several factors, including the quality of oocytes [35] and preparation of donor cell [25, 39-42]. In order to improve the SCNT efficiency, various strategies have been employed to modify the nuclear transfer procedure in an attempt to clone the dog successfully. Most of these efforts in canine SCNT are focused on preparation of donor cells. These include synchronizing the cell cycle stage of donor cells [25], modifying epigenetic marks of donor cells with chemical [42] and using somatic cells from donors of various passages [43] and culture conditions [40, 41].

Primary culture is the stage of the isolation of the cells from a piece of tissue and it is an indispensable procedure for SCNT to establish the donor cell. In primary culture, enzymes were used most frequently for tissue disaggregation since enzymatic disaggregation can yield a higher number of cells in a short time [7]. Among the various enzymes, trypsin was used as a common enzyme because it is easy to access and it could provide the most complete tissue disaggregation for starting primary cultures [7].

However, one of the disadvantages of using trypsin to disaggregate tissue is a risk of proteolytic damage to cells during enzymatic digestion under the optimal temperature (around 37 ℃) [7]. Therefore, it is widely assumed that treatment of

fibroblast with trypsin has some negative effects on cell viability [44]. Despite these negative effects, trypsin was commonly used during the primary culture procedure and trypsin-treated cells in the primary culture have been used as a donor cell for SCNT in various species, including cattle [45, 46], cat [47], goat [48] and buffalo [49].

In dog, there has been few studies about the effects of trypsin treatment on primary culture of canine cells and the subsequent efficiency of canine SCNT. Therefore, the aim of this study is to investigate the effect of trypsin treatment on primary culture and SCNT efficiency using trypsin-treated cells.

2. Materials and methods

2.1. Primary cell culture and preparation of donor cells

Skin tissue was isolated by an aseptic surgical method from the three dogs. The pieces of tissue were washed three times in Dulbecco's phosphate-buffered saline (DPBS; Invitrogen, Waltham, Massachusetts, USA) and minced in RCME-P medium with a surgical blade. Each minced skin tissue was divided into two groups (trypsin non-treated and trypsin-treated group) by similar weight respectively. To establish control (trypsin non-treated) group donor cells, the minced tissue was washed three times with RCME-P medium and seeded into plastic culture dishes (Becton Dickinson, Lincoln Park, NJ, USA) with RCME-P medium at 37 ℃ in a humidified atmosphere of 5% CO_2 and 95% air.

To establish trypsin-treated donor cells (trypsin-treated group), the minced tissue as in the control group was treated with 0.25% (w/v) trypsin ethylenediamine tetra-acetic acid (EDTA; Invitrogen) for 1 h at 37 ℃. The trypsinized tissue was washed three times in RCME-P medium and seeded into plastic culture dishes with RCME-P medium at 37 ℃ in 5% CO_2. After 6 to 8 days culture, unattached clumps of cells or explants were removed and the attached cells were further cultured to confluence. The cells were sub-cultured for further passaging, or stored in liquid nitrogen. The freezing medium consisted of 90% (v/v) FBS and 10% (v/v) Dimethyl sulfoxide (DMSO).

To prepare the donor cells for SCNT, the cells were thawed and cultured with DMEM supplemented with 10% (v/v) FBS. When it reached to confluence, cells

were retrieved just before the SCNT procedure by trypsinization for 3 min using 0.25% (w/v) trypsin supplemented with 1 mM EDTA, and suspended in PBS supplemented with 0.1% (v/v) FBS until used for SCNT. Donor cells from passage number 3 to 5 were used for SCNT.

2.2. Cell viability test using the Cell Counting Kit -8

To test a viability of cells, Cell Counting Kit-8 (CCK-8) solution (Dojindo, Kumamoto, Japan) was used. Briefly, control and trypsin-treated donor cells (2.0 x 10^4 cells/well) were plated with 200 μl of DMEM medium in 96-well for 24 h at 39 ℃ in 5% CO_2 to arrest mitosis. After 24 h, 10 μl of CCK-8 solution were added to each well and the plates were incubated for another 4 h at 39 ℃ in 5% CO_2. The absorbance was measured after 4h, at 450 nm using an ELISA spectrophotometer (Biorad Laboratories, Richmond, CA, USA). The control and trypsin-treated cells, which were derived from three dogs, were used for experiment through passage 1 to 4. Cell viability was measured at each passage and repeated more than four times.

2.3. Total RNA extraction and reverse transcription

The cell pellets from the trypsin-treated and control groups were harvested by trypsin-EDTA and rinsed with PBS for further analysis. Total RNAs were extracted from the cell pellet of both group using using the easy-spin Total RNA Extraction Kit (iNtRON bio Biotechnology Inc., Gyeonggi-do, Korea) according to the manufacturer's instructions with slight modifications where needed. To synthesize cDNA, 1 μg of total RNA was used with an amfiRivert cDNA Synthesis Platinum Master Mix (GenDEPOT Inc.) following the manufacturer's instructions. The

synthesized cDNAs were then stored at -20 ℃ until used for real-time PCR. All the products except total RNA were supplied with the kit.

2.4. Determination of cell apoptosis-related and growth-relative transcript levels by real-time PCR

Real-time PCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Each reaction mix was made with 2 μl of cDNA, 10 μl of SYBR Green Master Mix (Applied Biosystems), 4.4 μl of Nuclease-free water (Ambion Inc., Austin, TX, USA) and 1.8 μl each of forward and reverse primers. The sequences of primers and the sizes of amplified fragments from prohibitin (*PHB*), serine/threonine kinase 1 (*Akt 1*), phosphatidylinositol 3-kinase (*PI3K*), B cell lymphoma 2 (*Bcl2*) and Bcl2 associated X protein (*Bax*) are listed in Table 1. The control and trypsin-treated cells, which were derived from three dogs, were used for experiment through passage 1 to 4. Real-time PCR was performed at each passage and repeated at least five times.

2.5. Somatic cell nuclear transfer and transfer of cloned embryos

Recovery of *in vivo* matured oocytes was performed from oviducts approximately 72 h after ovulation. Cumulus cells from the *in vivo* oocytes were removed by repeated pipetting in 0.1% (v/v) hyaluronidase and oocytes were enucleated using micromanipulation. Control donor cells or trypsin-treated donor cells were introduced into the PVS of the enucleated oocytes, respectively. Then couplets were fused electrically and fused couplets were activated chemically. After activation, both cloned embryos were respectively surgically transferred into the

oviducts of naturally synchronous recipients [22, 35].

2.6. Statistical analysis

Levels of cell viability, relative transcript levels and SCNT efficiency were analyzed by a paired Student's *t*-test (GraphPad Prism version; Graphpad Incorporation, San Diego, CA, USA). These data were derived from experiments repeated at least four times.

Table 1. List of primers used for real-time PCR

Gene	Accession No.	Primer sequence (5'-3')	Product size (bp)
β-actin	NM_001003349	F- GCTACGTCGCCCTGGACTTC R- GCCCGTCGGGTAGTTCGTAG	86
PHB	XM_001110179	F- CCACCTCGGTGTCTCAAAAT R- CTCTGGAAGGGCAGTCTCTG	134
PI3K	NM_001287160	F- TCAGCCAAGCATTGTTGAAG R- GCACCAGCCGATCTACAAAT	90
Akt1	XM_014116176	F- GCAGGAGGAAGAGATGATGG R- CCCAGCAGCTTCAGGTACTC	135
Bax	NM_001003011	F- ACTTTGCCAGCAAACTGGTG R- AGGAAGTCCAGTGTCCAGCC	88
Bcl2	NM_001002949.1	F- TGAGTACCTGAACCGGCATC R- GTCAAACAGAGGCTGCATGG	100

3. Results

3.1. Effects of trypsin treatment on the number of cells in primary culture

In primary culture, the outgrowth of cells began in the trypsin-treated group about one day after explantation. In contrast, it started in the control group about two days after being seeded. As shown in Fig. 1, more cells were observed in the trypsin-treated group than in the control group after culture for five days and there were no differences in cell morphology. Primary cultured cells from both groups were harvested after day 11. The number of harvested cells within the same period of primary culture tended ($P = 0.14$) to increase in trypsin-treated group (Fig. 2).

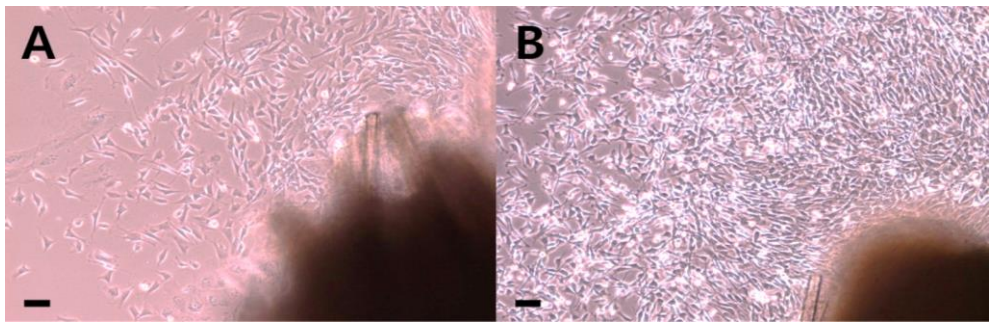

Fig. 1. Cells outgrowing on day 5 after primary culture, A) control group, B) trypsin-treated group (Scale bar = 10μm.).

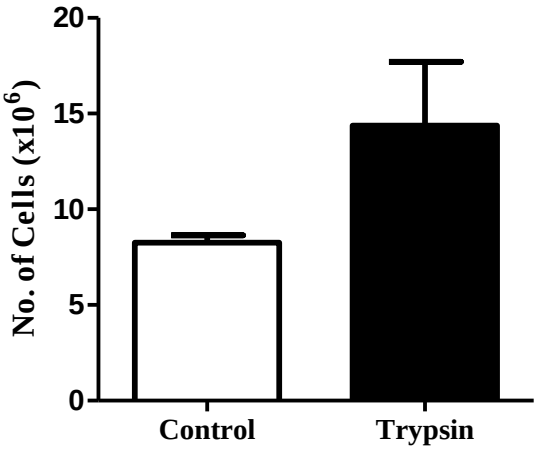

Fig. 2. Comparison of the cell number derived from primary culture between control and trypsin-treated groups (P = 0.14).

3.2. Effects of trypsin treatment on cell viability and the expression of apoptosis related transcripts

There were no significant differences between the control group (0.32 ± 0.03 Absorbance) and the trypsin-treated group (0.32 ± 0.02 Absorbance) (Fig. 3) with regard to cell viability using the CCK-8 kit. In addition, there was no significant difference in the transcripts levels of *Bax*/*Bcl2* ratio between the control and trypsin-treated groups (Fig. 4).

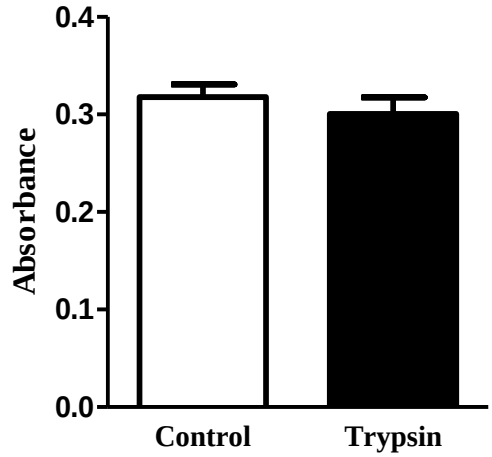

Fig. 3. Comparison of cell viability in control and trypsin-treated groups using CCK-8.

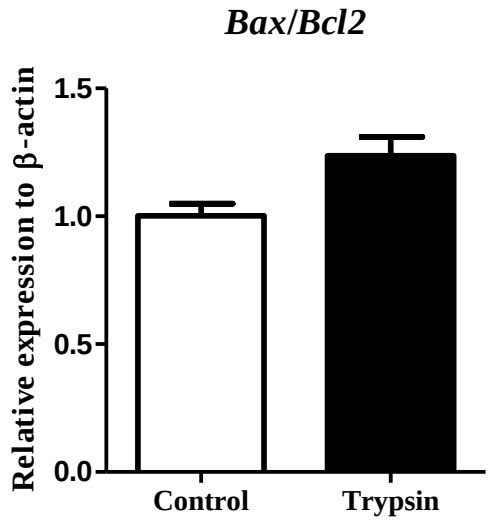

Fig. 4. Comparison of cell apoptosis-related transcript levels in control and trypsin-treated primary culture groups.

3.3. Expression of cell growth-related transcripts in donor cells treated with trypsin

The relative abundances of *PHB* were significantly increased to 1.6-fold in the trypsin-treated group compared to the control group. On the other hand, the transcripts levels of *Akt1* significantly decreased 1/5-fold in the trypsin-treated group and there was no difference between the control and trypsin-treated groups in *PI3K* transcript levels (Fig. 5).

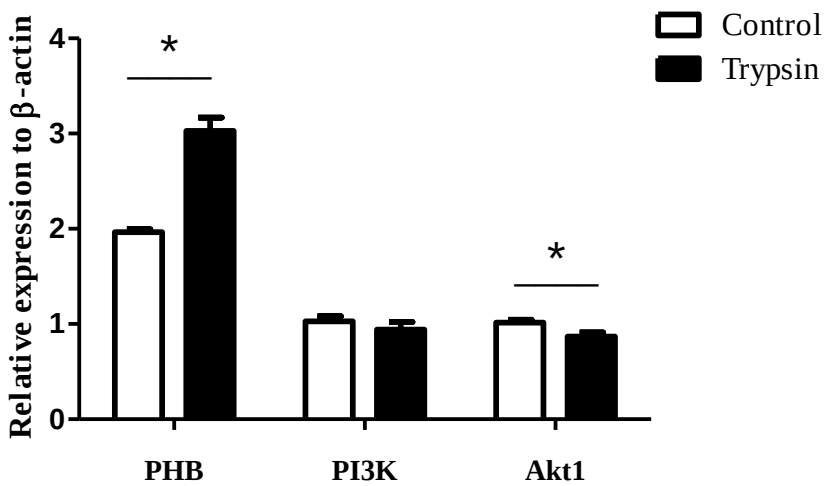

Fig. 5. Comparison of cell growth-related transcript levels in control and trypsin-treated primary culture groups. * means significantly difference ($P < 0.05$).

3.4. Effectiveness of trypsin-treated donor cells in SCNT

As shown in Table 2, a total of 90 SCNT embryos from the control group were surgically transferred into six surrogate dogs, and 71 reconstructed embryos using trypsin-treated group were transferred into five recipients. Three surrogate mothers in the control group became pregnant (50.0%) and also three in the trypsin-treated group (60.0%). Pregnancy was maintained to term and the three pregnant surrogates from the control group delivered four pups (4.4%) by Caesarean section, but two pups showed neck muscle abnormality, which frequently occurs in cloned dogs, and died within 2 days after birth due to breathing difficulties. The three pregnant surrogates from the trypsin-treated group also delivered four pups (5.6%) by Caesarean section but one of four pups also showed neck muscle abnormality and died on the same day (Fig. 6).

Table 2. *In vivo* development of cloned embryos produced by SCNT using control cells or trypsin-treated cells

Group	No. surrogates	No. transferred embryos	No. pregnancies (%)[a]	No. deliveries (%)[b]	No. abnormal puppies (%)[c]
Control	6	90	3 (50.0)	4 (4.4)	2 (50.0)
Trypsin	5	71	3 (60.0)	4 (5.6)	1 (25.0)

[a] Percentage based on the total number of recipient dogs.

[b] Percentage based on the total number of transferred embryos.

[c] Percentage based on the total number of delivered puppies.

Fig. 6. Picture of cloned dogs. A) Cloned dogs produced by SCNT using control cells. B) cloned dogs produced with trypsin-treated cells. C) cloned dogs which showed neck muscle abnormality.

4. Discussion

In this study, I demonstrated that 0.25% trypsin-EDTA treatment at 37 ℃ for 1 h of somatic cells in primary culture could upregulate *PHB* and downregulate *Atk1* transcript levels, which can block cell cycle. Also, the trypsin treatment made it possible to isolate a large number of cells and had no negative effect on cell viability, expression of *Bax*/*Bcl2* ratio and *in vivo* development of canine cloned embryos.

It is well known that trypsin is the most effective enzyme for dissociating tissue fragments and aggregating into a single cell suspension [50]. A previous study showed that trypsin cytotoxicity reduces cell viability under various conditions (batches of trypsin, trypsin concentrations and incubation duration) [51]. Consistent with the above study, our results also showed negatively affected cell growth-related genes by trypsin treatment during primary culture. *PHB*, the member of the Band-7 family, has important biological roles in cell proliferation, cell development and mitochondrial function. It is a negative regulator of cell-cycle progression [52]. In human fibroblasts, cell-cycle progression was suppressed by microinjection of *PHB* mRNA which blocked cells to enter the G1 to S-phase [52, 53]. The *PI3K*/*Akt* pathway is a signal transduction pathway that encourages cell survival and growth [54]. Our results showed that there was no significant difference between the trypsin treatment and control groups in *PI3K* gene transcript levels. On the contrary, trypsin treatment significantly increased *PHB*, and decreased *Akt* transcript levels. The expression levels of *PHB* [55] and *Akt* [56] were used to expect the cell cycle.

Therefore, these results indicated that the cell cycle might be suppressed since the trypsin treatment upregulated *PHB* and downregulated *Akt*.

Nevertheless, I found that 0.25% trypsin-EDTA treatment at 37 ℃ for 1 h did not influence cell viability and expression of *Bax*/*Bcl2* ratio in canine skin tissue primary culture. The proapoptotic gene *Bax* and the antiapoptotic gene *Bcl2* are critical determinants of the apoptotic response. Therefore, *Bax*/*Bcl2* ratio is commonly used as an indicator of apoptosis [57]. At the same time, cell numbers in trypsin treatment group had a tendency ($P = 0.14$) to increase compared to the control group. This result was consistent with the previous study that proliferative capacity in primary culture was higher when keratinocytes from human skin were harvested by trypsin treatment [58]. It is established that trypsin treatment completely disaggregates tissues during primary culture.

Furthermore, using trypsin-treated cells during primary culture as donor cells for SCNT showed no negative effects on pregnancy and delivery rates compared with the control group cells. Both control and trypsin-treated groups showed similar proportion of pregnancy and delivery, indicating that using trypsin-treated cells as donor cells has no trouble cloning the dogs. Birth defects in cloned dogs were observed in this study, which might be due to aberrant epigenetic reprogramming during the SCNT. Up to now, there were some reports on developmental abnormalities in cloned mammals including dogs [59, 60]. In addition, both cloned dogs from control cells and trypsin-treated cells showed the developmental defects, indicating that trypsin treatment did not contribute to the negative effect on birth defects during primary culture.

In conclusion, somatic cells established by trypsin treatment during primary culture showed no negative consequences in cell viability, expression of *Bax*/*Bcl2* ratio and canine SCNT efficiency. Compared to the control group, trypsin treatment had a trend (P = 0.14) to harvest more numbers of somatic cells in the same period. These findings will be useful for cloning researches using of trypsin during primary culture to establish the cell lines for SCNT.

PART IV

CLONING OF SHORT TAIL GYEONG-JU DONGGYEONG DOG *VIA* SCNT; CONSERVING PHENOTYPIC INHERITANCE

1. Introduction

Since the production of the first cloned dog, Snuppy (Afghan Hound) [22], several species such as beagle [39], toy poodle [24], Retriever [25], Border collie [61] and Pekingese [62] have been obtained by somatic cell nuclear transfer (SCNT). Among many breeds which needed to be conserved from extinction, Sapsaree, one of Korean natural monument dogs, has been cloned by SCNT [63].

The Gyeong-ju Donggyeong dog has been considered as a natural monument since 2012 (Cultural Heritage Administration of Korea, number: 540). The name of Gyeong-ju Donggyeong dog originated from the capital of ancient Silla kingdom in Korea. The Donggyeong dog has the oldest history among the Korean natural monument breeds, and it is referred to in plenty of historic documents, such as Dongkyung jabki (published in AD 1669) and Sungho sasul (published in AD 1740). Despite the Donggyeong dog's high historical value, only about two hundred individuals remain in Gyeong-ju, and it is classified as endangered [1-3]. For this reason, it is essential to conserve such valuable breed from extinction and maintain a pure descent.

Accordingly, the purpose of this study was to produce the Donggyeong dog by SCNT and observe the similarity of phenotypes between the cloned dog and the cell donor dog.

2. Materials and methods

2.1. Primary culture and preparation of donor cells

Skin tissue was isolated by aseptic surgical method from a 3-month-old female short-tailed Donggyeong dog. The pieces of tissue were washed three times in DPBS and minced in RCME-P medium. The minced tissue were treated with 0.25% (w/v) trypsin-EDTA for 1 h at 37 ℃. Trypsinized tissues were washed three times in RCME-P medium by centrifugation at 1500 rpm for 2 min and seeded into plastic culture dishes. After 6 to 8 days culture, unattached clumps of cells or explants were removed and the attached cells were further cultured to confluence. The cells were maintained in culture or cryopreserved in 90% (v/v) FBS and 10% (v/v) DMSO and stored in liquid nitrogen.

To prepare the donor cells for SCNT, the cells were thawed and washed two times in DMEM supplemented with 10% (v/v) FBS and cultured with DMEM supplemented with 10% (v/v) FBS. When the cells reached to confluence, cells were retrieved by trypsinization for 3 min using 0.25% (w/v) trypsin supplemented with 1 mM EDTA, and then cells were suspended in PBS supplemented with 0.1% (v/v) FBS. Donor cells from passage number 2 to 5 were used for SCNT [22].

2.2. DNA extraction and microsatellite analysis

After cloned dog were born, microsatellite analyses were performed. Parentage analysis was performed on the nuclear donor, cloned dog, oocyte donor dogs and surrogate recipient. Blood samples were collected from the recipients and

cloned Donggyeong dog. For donor Donggyeong dog, cultured fibroblasts were used and skin tissue of the oocytes donor were obtained for genomic DNA extraction. Genomic DNA from blood, donor cells and skin tissue was extracted according to instructions supplied with the G-spin™ Genomic DNA Extraction Kit (Intron, Seoul, Korea). The following 6 markers were selected for analysis: PEZ2, PEZ10, PEZ16, PEZ17, CPH4 and CPH12. Isolated genomic DNA samples were dissolved in 50 ml TE and used for microsatellite analysis with 6 specific markers. Length variations were assayed by polymerase chain reaction (PCR) amplification with fluorescently labeled locus-specific primers and PAGE on an automated DNA sequencer (ABI; Applied Biosystems, Foster City, CA, USA). Proprietary software (GeneScan and Genotyper; Applied Biosystems) was used to estimate the PCR product size of nucleotides.

2.3. PCR amplification and mitochondrial DNA analysis

To identify the origin of the mitochondrial DNA (mtDNA) in cloned dog, the previously extracted genomic DNA were used canine mtDNA (GenBank accession no U96639 v.2, and 650 bases) analysis. The PCR amplification was conducted in a 50 μl volume, including 5 μl of 10X reaction buffer (1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 μM primer, 1.5 U Taq DNA polymerase and 30-50 ng of genomic DNA). Products of the PCR were purified by a Power Gel Extraction Kit (Takara Biosystems, Shiga, Japan) and sequenced with an ABI3100 instrument (Applied Biosystems) and mtDNA were confirmed by BLAST search.

2.4. Radiographic measurements

To compare the number of coccygeal vertebral bodies of the cell donor dog (six months old) and cloned dog (20 days after birth), ventrodorsal radiographic assessments were performed with digital radiographic system (EVA-HF 525, COMED medical system, Seongnam, Korea). Ventrodorsal projection of the pelvis were performed using 57 Kilovolt peak (kVp) and 4.0 milliampere-seconds (mAs) for cell donor dog and 30 kVp and 2.7 mAs for cloned dog. All radiographs were obtained with a standard 100 cm beam source distance. The dog was placed in dorsal recumbency in a positioning V-trough and the pelvic limbs have been extended and hold at the level of the stifle joints for appropriate positioning of the pelvic limbs relative to the pelvis and table [64]. Field of view was placed iliac crest to the end of coccygeal vertebral bodies. The coccygeal vertebral number was measured as the number from the dorsal surface of the sacrum.

3. Results

3.1. *In vivo* development of cloned embryos using somatic cells derived from Donggyeong dog

In total, 42 reconstructed embryos, using trypsin donor cells from six oocyte donor dogs were also transferred to three surrogate dogs. One recipient (33.3%) was confirmed pregnant by ultrasonography 23 days after embryo transfer. Pregnancy was maintained to term and one healthy female Donggyeong dog weighing 320g were delivered (2.3%) by caesarean section on Day 59 (Table 3).

Table 3. *In vivo* development of cloned embryos by SCNT using somatic cells derived from donor dog

Recipient	No. oocytes donors	Oocyte status [a]	No. reconstructed couplets	Pregnancy	No. cloned dogs
A	2	Mature	15	-	-
B	1	Mature	6	-	-
C	3	Mature and early aged	21	+	1
Total (n = 3)	6		42	1 (33.3%) [b]	1 (2.3%) [c]

[a] Status of *in vivo* oocytes flushed from oviducts approximately 72 h after ovulation.

[b] Percentage was based on the total number of recipient dogs.

[c] Percentage was based on the total number of transferred embryos.

Fig. 7. Pictures of the cell donor and cloned Donggyeong dogs. A) cell donor dog at three months old. B) cloned dog at 1 day after birth. C) tail length of cell donor dog. D) tail length of cloned dog.

3.2. Microsatellite analysis and mitochondrial DNA analysis of cloned Donggyeong dog

Based on the results, I identified that the cloned dog had identical mtDNA sequences to those of the domestic oocyte donor and recipient dog (Table 4). Furthermore, to determine parentage, DNA extraction and microsatellite analysis with canine-specific markers were performed following the protocol of our previous study [35]. Based on the results of the parentage analysis indicated that the cloned Donggyeong dog was genetically identical to the cell donor Donggyeong dog (Table 5).

Table 4. Sequence alignments within 628 bases of the hypervariable region of mitochondrial DNA

Sample ID	Nucleotide positions*																		
	15518	15526	15595	15612	15627	15632	15639	15643	15652	15665	15800	15814	15815	15912	15955	16003	16025 16	16083	16105
Reference	A	C	C	T	A	C	T	A	G	T	T	C	T	C	C	A	T	A	T
Cell donor	A	C	C	T	A	C	A	A	G	T	T	T	T	C	C	A	C	A	T
Cloned dog	A	C	C	T	G	C	A	A	A	C	T	T	T	C	C	A	T	A	T
Surrogate	A	C	C	T	G	C	A	A	A	C	T	T	T	C	C	A	T	A	T
Oocytes donor 1	A	C	C	T	G	C	A	A	A	C	T	T	T	C	C	A	T	A	T
Oocytes donor 2	A	C	C	T	G	C	A	A	A	C	T	T	T	C	C	A	T	A	G
Oocytes donor 3	C	T	T	C	A	T	G	G	A	T	C	T	C	T	T	G	T	G	T

*The nucleotide positions were numbered according to GenBank accession no. U96639 v.2, and 650 bases (from 15461 to 16110) were examined.

Table 5. Microsatellite genotyping of cell donor, cloned, surrogate and oocytes donor dogs using specific canine DNA markers

NAME	Cell donor	Cloned	Surrogate	Oocyte donor 1
PEZ2	130 / 130	130 / 130	126 / 122	126 / 126
PEZ10	298 / 282	298 / 282	282 / 282	282 / 262
PEZ16	298 / 290	298 / 290	302 / 286	302 / 282
CPH4	149 / 137	149 / 137	141 / 141	141 / 141
PEZ17	222 / 214	222 / 214	218 / 202	210 / 210
CPH12	207 / 207	207 / 207	203 / 193	193 / 193

3.4. Comparison of the coccygeal vertebra of cell donor dog and a cloned dog using x-ray

To identify the number of caudal vertebral bodies, the dorsal radiographic views of the caudal vertebral column in the cloned dog and donor dog were compared. The number of coccygeal vertebral bodies was counted from the sacrum of the dorsal surface of the vertebral body. Commonly, dog has 6 to 23 of coccygeal vertebral column but the cell donor Donggyeong dog had six coccygeal vertebral bodies and the cloned Donggyeong dog had seven coccygeal vertebral bodies (Fig. 8 A, B).

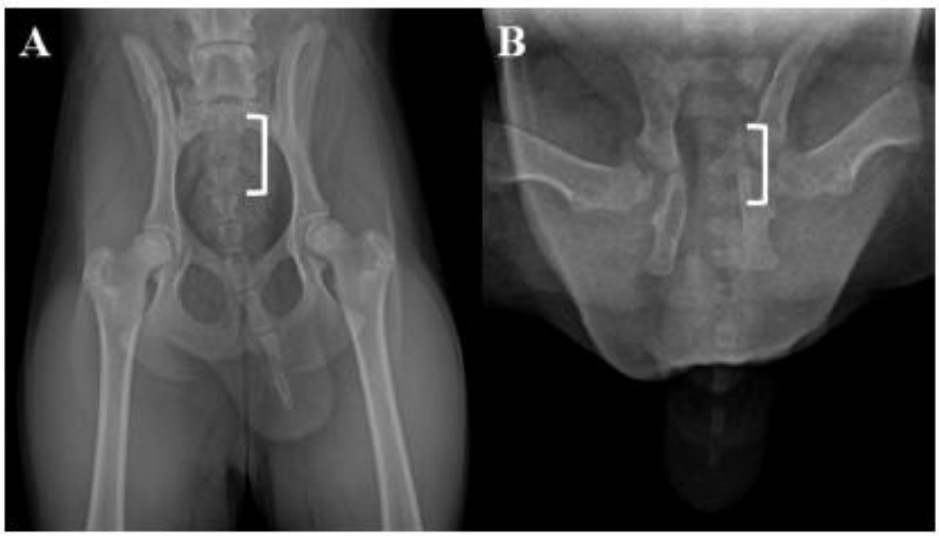

Fig. 8. Comparison of the number of coccygeal vertebral bodies of the cloned Donggyeong dog (20 days after birth) and a donor Donggyeong dog (six months old) using digital radiographic views. A) a dorsal radiographic view of a portion of the caudal vertebral column of a cell donor dog is shown to illustrate measurements obtained for the sacrum (white bracket) through to the last coccygeal vertebra. B) dorsal radiographic view of the cloned dog.

4. Discussion

In this study, a Donggyeong dog, which is considered an endangered breed and essential to be saved from extinction, was cloned by SCNT for the first time. The cloned Donggyeong dog had identical mtDNA sequences to those of the domestic oocyte donor and recipient dog (Table 5). Since the oocyte donor dog and surrogate dog had the same information for mtDNA in the mtDNA analysis, it is difficult to distinguish which dog's mtDNA was transferred to the cloned dog (Table 6). Many studies about dog cloning proved that the cloned dog's mtDNA was transferred solely from the oocyte donor [24, 61, 63]. Similarly, in this study, the mtDNA of the cloned dog might have been transferred from the oocyte donor dog.

It has been reported that a cloned toy poodle had the same coat color as the somatic cell donor dog [24] and that beagles, which cloned from fetal fibroblasts had similar coat spotting [39]. In addition, a cloned Sapsaree had hip dysplasia, which is a genetic disease, and this was also founded in the cell donor Sapsaree [29]. In the present study, the cloned Donggyeong dog also had a phenotype similar to that of the cell donor Donggyeong dog. The unique feature of the Donggyeong dog is that it can be classified as natural short tailed or tailless. According to the information from the Korean Gyeong-ju Donggyeong Dog Association, the short tailed Donggyeong dog (over twelve months old) has a tail length of around 11.38 ± 2.44 cm and 5 to 9 of coccygeal vertebrae in radiographic observations [2, 3]. The tailless Donggyeong dog has 1 to 4 of coccygeal vertebrae, and adult dogs (over twelve months old) have a tail length of around 6.3 ± 2.81 cm.

In this study, even though cell donor Donggyeong dog and cloned Donggyeong dogs had different numbers of coccygeal vertebral bodies, they could both be categorized as short-tailed Donggyeong dogs. It has been reported that despite cloned dogs having the same genetic information, they can have different dental development; one cloned dog had normal dental formulas, another cloned dog was missing one permanent molar tooth on the left side, and another cloned dog was missing one permanent molar tooth on the right side [65]. I cannot explain the reason for these differences between a donor and a cloned dogs, but they might be associated with epigenetic modification during the cloning procedure.

The current study demonstrated that SCNT could not only be used for conserving a specific breed of dog but also that it could ensure inheritance of a unique phenotypic feature of a dog breed. To identify the relationship between the coccygeal vertebrae and epigenetic modification, further studies need to analyze epigenetic mechanisms, which can influence coccygeal vertebra development.

PART V

FINAL CONCLUSION

This thesis was conducted to clone the Gyeong-ju Donggyeong dog, endangered natural monument, using trypsin-treated cells during the primary culture. To investigate the effect of trypsin treatment on primary culture, I compared isolated cell numbers, viability of harvested cells, transcript levels of cell growth-related and apoptosis-related genes and cloning efficiency between control and trypsin-treated groups. Trypsin treatment in primary culture had a tendency ($P = 0.14$) to increase the somatic cell numbers and did not affect cell viability, expression of *Bax/Bcl2* ratio and canine SCNT efficiency. Based on these results, cloning of the Gyeong-ju Donggyeong dog were performed using the donor cells derived from trypsin-treated primary culture. One Donggyeong dog was cloned by SCNT and cloned puppy had the same genetic information with nuclear donor dog and short tail, the unique phenotype of the Donggyeong dog.

In conclusion, trypsin treatment on primary culture had no negative effects on cell viability and canine SCNT efficiency and the number of isolated cells tended to increase in trypsin-treated group. Furthermore, SCNT could not only be used for conserving a specific breed of dog but also for ensuring the inheritance of a unique phenotypic feature of a dog breed.

REFERENCES

[1] Choi SG. Studies on the origin and breed characteristics of Gyeongju Donggyeong dog. PhD Dissertation Daegu University, Korea. 2010.

[2] Choi SG, Sung KC, Lee EW. Study on anatomical characteristics by radiographic evaluation of the coccyx and pelvis in the Gyeongju Donggyeong dogs. Research of Gyeongju. 2010;19:163-73.

[3] Park CE, Lee EW, Sung KC, Choi SG. Investigation of candidated genes for molecular characterization of Donggyeong dog populations (Gyeongju). The Korean Society of Veterinary Service. 2010;33:361-6.

[4] Freshney RI. Culture of animal cells : a manual of basic technique and specialized applications. 6th ed. Hoboken, N.J.: Wiley-Blackwell; 2010.

[5] Harrison RG. Observations on the living developing nerve fiber. Proc Soc Exp Biol Med. 1907:140–3.

[6] Carrel A. On the Permanent Life of Tissues Outside of the Organism. J Exp Med. 1912;15:516-28.

[7] Freshney RI. Culture of animal cells : a manual of basic technique. 5th ed. Hoboken, N.J.: Wiley-Liss; 2005.

[8] Waymouth C. To disaggregate or not to disaggregate injury and cell disaggregation, transient or permanent? In Vitro. 1974;10:97-111.

[9] Cole RJ, Paul J. The effects of erythropoietin on haem synthesis in mouse yolk sac and cultured foetal liver cells. J Embryol Exp Morphol. 1966;15:245-60.

[10] Schaffer K, Herrmuth H, Mueller J, Coy DH, Wong HC, Walsh JH, et al. Bombesin-like peptides stimulate somatostatin release from rat fundic D cells in primary culture. Am J Physiol. 1997;273:G686-95.

[11] Glavin GB, Szabo S, Johnson BR, Xing PL, Morales RE, Plebani M, et al. Isolated rat gastric mucosal cells: optimal conditions for cell harvesting, measures of viability and direct cytoprotection. J Pharmacol Exp Ther. 1996;276:1174-9.

[12] Freshney RI. Tumour cells disaggregated in collagenase. Lancet. 1972;2:488-9.

[13] Speirs V, White MC, Green AR. Collagenase III: a superior enzyme for complete disaggregation and improved viability of normal and malignant human breast tissue. In Vitro Cell Dev Biol Anim. 1996;32:72-4.

[14] Compton CC, Warland G, Nakagawa H, Opitz OG, Rustgi AK. Cellular characterization and successful transfection of serially subcultured normal human esophageal keratinocytes. J Cell Physiol. 1998;177:274-81.

[15] Inamatsu M, Matsuzaki T, Iwanari H, Yoshizato K. Establishment of rat dermal papilla cell lines that sustain the potency to induce hair follicles from afollicular skin. J Invest Dermatol. 1998;111:767-75.

[16] Lasfargues EY, Coutinho WG, Lasfargues JC, Moore DH. A serum substitute that can support the continuous growth of mammary tumor cells. In Vitro. 1973;8:494-500.

[17] Zaroff L, Sato, G. H.,&Mills, S. E. Single-cell platings from freshly isolated mammalian tissue. Exp Cell Res. 1961:565–75.

[18] Brown WE, Wold F. Alkyl isocyanates as active-site-specific reagents for serine proteases. Identification of the active-site serine as the site of reaction. Biochemistry. 1973;12:835-40.

[19] Soleimani M, Nadri S. A protocol for isolation and culture of mesenchymal stem cells from mouse bone marrow. Nat Protoc. 2009;4:102-6.

[20] Banumathi E, Haribalaganesh R, Babu SS, Kumar NS, Sangiliyandi G. High-yielding enzymatic method for isolation and culture of microvascular endothelial cells from bovine retinal blood vessels. Microvasc Res. 2009;77:377-81.

[21] Yang M, Chen CZ, Wang XN, Zhu YB, Gu YJ. Favorable effects of the detergent and enzyme extraction method for preparing decellularized bovine pericardium scaffold for tissue engineered heart valves. J Biomed Mater Res B Appl Biomater. 2009;91:354-61.

[22] Lee BC, Kim MK, Jang G, Oh HJ, Yuda F, Kim HJ, et al. Dogs cloned from adult somatic cells. Nature. 2005;436:641.

[23] Park J, Oh H, Hong S, Kim M, Kim G, Koo O, et al. Effective donor cell fusion conditions for production of cloned dogs by somatic cell nuclear transfer. Theriogenology. 2011;75:777-82.

[24] Jang G, Hong SG, Oh HJ, Kim MK, Park JE, Kim HJ, et al. A cloned toy poodle produced from somatic cells derived from an aged female dog. Theriogenology. 2008;69:556-63.
[25] Oh HJ, Hong SG, Park JE, Kang JT, Kim MJ, Kim MK, et al. Improved efficiency of canine nucleus transfer using roscovitine-treated canine fibroblasts. Theriogenology. 2009;72:461-70.
[26] Oh HJ, Choi J, Kim MJ, Kim GA, Jo YK, Choi YB, et al. Propagation of elite rescue dogs by somatic cell nuclear transfer. Anim Sci J. 2016;87:21-6.
[27] Kim MK, Jang G, Oh HJ, Yuda F, Kim HJ, Hwang WS, et al. Endangered wolves cloned from adult somatic cells. Cloning Stem Cells. 2007;9:130-7.
[28] Oh HJ, Kim MK, Jang G, Kim HJ, Hong SG, Park JE, et al. Cloning endangered gray wolves (Canis lupus) from somatic cells collected postmortem. Theriogenology. 2008;70:638-47.
[29] Jang G, Hong S, Kang J, Park J, Oh H, Park C, et al. Conservation of the Sapsaree (Canis familiaris), a Korean Natural Monument, using somatic cell nuclear transfer. J Vet Med Sci. 2009;71:1217-20.
[30] Hong SG, Kim MK, Jang G, Oh HJ, Park JE, Kang JT, et al. Generation of red fluorescent protein transgenic dogs. Genesis. 2009;47:314-22.
[31] Kim MJ, Oh HJ, Park JE, Kim GA, Hong SG, Jang G, et al. Generation of transgenic dogs that conditionally express green fluorescent protein. Genesis. 2011;49:472-8.
[32] Holt WV, Pickard AR. Role of reproductive technologies and genetic resource banks in animal conservation. Rev Reprod. 1999;4:143-50.
[33] Andrabi SM, Maxwell WM. A review on reproductive biotechnologies for conservation of endangered mammalian species. Anim Reprod Sci. 2007;99:223-43.
[34] Comizzoli P, Mermillod P, Mauget R. Reproductive biotechnologies for endangered mammalian species. Reprod Nutr Dev. 2000;40:493-504.
[35] Jang G, Kim MK, Oh HJ, Hossein MS, Fibrianto YH, Hong SG, et al. Birth of viable female dogs produced by somatic cell nuclear transfer. Theriogenology. 2007;67:941-7.

[36] Zakhartchenko V, Mueller S, Alberio R, Schernthaner W, Stojkovic M, Wenigerkind H, et al. Nuclear transfer in cattle with non-transfected and transfected fetal or cloned transgenic fetal and postnatal fibroblasts. Mol Reprod Dev. 2001;60:362-9.

[37] Boquest AC, Grupen CG, Harrison SJ, McIlfatrick SM, Ashman RJ, d'Apice AJ, et al. Production of cloned pigs from cultured fetal fibroblast cells. Biol Reprod. 2002;66:1283-7.

[38] Wakayama T, Yanagimachi R. Mouse cloning with nucleus donor cells of different age and type. Mol Reprod Dev. 2001;58:376-83.

[39] Hong SG, Jang G, Kim MK, Oh HJ, Park JE, Kang JT, et al. Dogs cloned from fetal fibroblasts by nuclear transfer. Anim Reprod Sci. 2009;115:334-9.

[40] Kim GA, Oh HJ, Lee TH, Lee JH, Oh SH, Lee JH, et al. Effect of culture medium type on canine adipose-derived mesenchymal stem cells and developmental competence of interspecies cloned embryos. Theriogenology. 2014;81:243-9.

[41] Kim GA, Oh HJ, Kim MJ, Jo YK, Choi J, Kim JW, et al. Effect of primary culture medium type for culture of canine fibroblasts on production of cloned dogs. Theriogenology. 2015;84:524-30.

[42] Kim MJ, Oh HJ, Kim GA, Suh HN, Jo YK, Choi YB, et al. Altering histone acetylation status in donor cells with suberoylanilide hydroxamic acid does not affect dog cloning efficiency. Theriogenology. 2015;84:1256-61.

[43] Oh HJ, Park EJ, Lee SY, Soh JW, Kong IS, Choi SW, et al. Comparison of cell proliferation and epigenetic modification of gene expression patterns in canine foetal fibroblasts and adipose tissue-derived mesenchymal stem cells. Cell Prolif. 2012;45:438-44.

[44] McKeehan WL. The effect of temperature during trypsin treatment on viability and multiplication potential of single normal human and chicken fibroblasts. Cell Biol Int Rep. 1977;1:335-43.

[45] Pan S, Chen W, Liu X, Xiao J, Wang Y, Liu J, et al. Application of a novel population of multipotent stem cells derived from skin fibroblasts as donor cells in bovine SCNT. PLoS One. 2015;10:e0114423.

[46] Cho SR, Ock SA, Yoo JG, Mohana Kumar B, Choe SY, Rho GJ. Effects of confluent, roscovitine treatment and serum starvation on the cell-cycle synchronization of bovine foetal fibroblasts. Reprod Domest Anim. 2005;40:171-6.
[47] Kitiyanant Y, Saikhun J, Pavasuthipaisit K. Somatic cell nuclear transfer in domestic cat oocytes treated with IGF-I for in vitro maturation. Theriogenology. 2003;59:1775-86.
[48] Zhang LS, Jiang MX, Lei ZL, Li RC, Sang D, Sun QY, et al. Development of goat embryos reconstituted with somatic cells: the effect of cell-cycle coordination between transferred nucleus and recipient oocytes. J Reprod Dev. 2004;50:661-6.
[49] Deng Y, Liu Q, Luo C, Chen S, Li X, Wang C, et al. Generation of induced pluripotent stem cells from buffalo (Bubalus bubalis) fetal fibroblasts with buffalo defined factors. Stem Cells Dev. 2012;21:2485-94.
[50] Gray DH, Chidgey AP, Boyd RL. Analysis of thymic stromal cell populations using flow cytometry. J Immunol Methods. 2002;260:15-28.
[51] Takashima A. Establishment of fibroblast cultures. Curr Protoc Cell Biol. 2001;Chapter 2:Unit 2 1.
[52] Mishra S, Murphy LC, Nyomba BL, Murphy LJ. Prohibitin: a potential target for new therapeutics. Trends Mol Med. 2005;11:192-7.
[53] Nuell MJ, Stewart DA, Walker L, Friedman V, Wood CM, Owens GA, et al. Prohibitin, an evolutionarily conserved intracellular protein that blocks DNA synthesis in normal fibroblasts and HeLa cells. Mol Cell Biol. 1991;11:1372-81.
[54] Osaki M, Oshimura M, Ito H. PI3K-Akt pathway: its functions and alterations in human cancer. Apoptosis. 2004;9:667-76.
[55] Kathiria AS, Butcher MA, Hansen JM, Theiss AL. Nrf2 is not required for epithelial prohibitin-dependent attenuation of experimental colitis. Am J Physiol Gastrointest Liver Physiol. 2013;304:G885-96.
[56] Tankiewicz-Kwedlo A, Hermanowicz J, Surazynski A, Rozkiewicz D, Pryczynicz A, Domaniewski T, et al. Erythropoietin accelerates tumor growth through increase of erythropoietin receptor (EpoR) as well as by the stimulation of angiogenesis in DLD-1 and Ht-29 xenografts. Mol Cell Biochem. 2016;421:1-18.

[57] Oltvai ZN, Milliman CL, Korsmeyer SJ. Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell. 1993;74:609-19.
[58] Hybbinette S, Bostrom M, Lindberg K. Enzymatic dissociation of keratinocytes from human skin biopsies for in vitro cell propagation. Exp Dermatol. 1999;8:30-8.
[59] Kim MJ, Kang SC, Kim JH, Oh HJ, Kim GA, Jo YK, et al. Ectopic liver and gallbladder in a cloned dog: Possible nonheritable anomaly. Theriogenology. 2015;84:995-1002.
[60] Kim MJ, Oh HJ, Kim GA, Jo YK, Choi J, Kim HJ, et al. Reduced birth weight, cleft palate and preputial abnormalities in a cloned dog. Acta Vet Scand. 2014;56:18.
[61] Kim GA, Oh HJ, Park JE, Kim MJ, Park EJ, Lim SH, et al. Employing mated females as recipients for transfer of cloned dog embryos. Reprod Fertil Dev. 2013;25:700-6.
[62] Park JE, Oh HJ, Hong SG, Kim MJ, Kim GA, Koo OJ, et al. Effective donor cell fusion conditions for production of cloned dogs by somatic cell nuclear transfer. Theriogenology. 2011;75:777-82.
[63] Jang G, Hong SG, Kang JT, Park JE, Oh HJ, Park CK, et al. Conservation of the Sapsaree (Canis familiaris), a Korean natural monument, using somatic cell nuclear transfer. J Vet Med Sci. 2009;71:1217-20.
[64] David. J. CR. Small Animal Pelvic Radiography. J NAVC. 2011;2:48-53.
[65] Park JE, Kim MK, Kang JT, Oh HJ, Hong SG, Kim DY, et al. Growth and hematologic characteristics of cloned dogs derived from adult somatic cell nuclear transfer. Cell Reprogram. 2010;12:141-50.

국문초록

일차배양시 트립신을 처리한 세포를 이용한 경주개 동경이의 복제

최 유 빈

(지도교수: 이 병 천)

서울대학교 대학원

수의학과 임상수의학 전공

체세포핵이식은 동물의 유전적 정보의 유지에 유용한 기술로 생각 되며 멸종 위기 동물의 종 보존에 있어 응용이 가능 한 것 또한 입증되었다. 경주개 동경이는 2012 년 천연기념물로 지정 되었으며, 개과 천연기념물 중 가장 오래된 역사를 가지고 있다. 그러나 유전적으로 가치가 있는 동경이는 일제강점기서부터 개체수가 급속도로 감소 하였다. 최근에는 약 400 여마리만이 경주 지역에 서식하고 있으며 지속적인 개체관리가 필요로 하여

진다. 이러한 천연기념물을 멸종으로부터 보호하고 순수혈통을 보존하는 것이 필수적이다.

체세포핵이식에 있어 일차배양은 세포주 확립에 있어 필수적인 단계이다. 그러나 멸종위기종의 경우 일차배양에 필요로 하는 조직을 회수 하는 것에 어려움이 있다. 그러므로 한정적인 양의 조직으로 많은 양의 체세포를 확보하는 일차 배양 방법을 확립 하는 것이 필요하다. 트립신은 일차배양시 체세포를 빠르게 회수 하기 위해 널리 이용되고 있다. 그러나 트립신이 일차배양에 미치는 영향과 일차배양시 트립신을 처리한 세포를 이용하여 체세포핵이식을 진행했을 경우 체세포핵이식 효율에 미치는 영향에 관한 연구가 많이 진행되어 있지 않다.

일차 배양시 트립신을 처리하는 것이 미치는 영향을 알아보기 위해, 본 연구에서는 일차배양시 트립신을 처리하지 않은 비처리군과 트립신 처리군 간의 확립된 세포수, 세포 생존능, 세포사와 세포 성장관련 유전자의 발현 정도 그리고 두 군의 세포를 이용하여 생산한 복제 배아의 체내 발달 여부를 비교하였다. 일차배양시 트립신을 처리한 공여 세포주 (트립신 처리군)를 확립하기 위해서 배양 전의 피부 조직 조각을 0.25%

트립신-에틸렌디아민테트라아세트산과 함께 1 시간간 37 ℃ 에서 처리하였고 다른 군은 처리하지 않았다 (비 처리군).

결과적으로 같은 기간 동안의 일차 배양시, 트립신을 처리하는 것이 많은 세포가 확립되는 경향을 보였다. 그러나 트립신 처리군과 비처리군 사이의 세포 생존능과 세포사 관련 유전자의 발현 정도의 차이는 없었다. 세포 성장 관련 유전자 발현 정도는 트립신 처리군이 비 처리군에 비해 유의적으로 높은 *PHB* 의 별현과 유의적으로 낮은 *Akt1* 의 발현을 보였다. 위의 실험에 사용한 세 쌍의 비 처리군과 트립신 처리군 중 한 쌍의 비 처리군과 트립신 처리군 세포를 체세포핵이식 과정에 사용 하여 복제 효율을 비교하였다. 트립신 처리군은 90 개의 복제 배아가 여섯 마리의 대리모에 이식 되었고 비처리군의 경우 71 개의 복제 배아가 여섯마리의 대리모에 이식 되었다. 각 그룹 모두 3 마리씩의 대리모가 임신이 확인되었고 (비 처리군; 50.0% 대 트립신 처리군; 60.0%), 각 그룹 4 마리씩의 산자가 태어났다 (비 처리군; 4.4% 대 트립신 처리군; 5.6%).

이전의 실험 결과를 토대로 동경이의 세포주 확립을 위한 일차 배양시 피부 조직을 트립신에 처리하였다. 확립된 세포를 이용하여 체세포핵이식을 진행하였고, 총 42 개의 복제 배아를

세마리의 대리모에 이식하였다. 한 마리의 대리모가 임신한 것이 확인되었고 (33.3%) 한 마리의 동경이가 제왕절개로 태어났다 (2.3%). 복제된 동경이는 세포공여 동경이와 유전적으로 동일하였으며, 동경이의 특별한 특징 중 하나인 단미를 가지고 있었다.

결론적으로, 일차 배양시 트립신을 처리 하는 것은 세포의 생존능과 세포사 관련 유전자의 발현 정도, 개의 체세포핵이식 효율에 영향을 주지 않았고, 이 방법은 비 처리 방법과 비교 하였을 때 증가된 세포 수를 보이는 추세였다. 본 연구는 최초로 천연기념물 경주개 동경이의 복제에 성공 하였고, 동경이의 특유의 짧은 꼬리 길이가 체세포핵이식을 통하여 보존 된다는 것을 증명하였다.

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주요어: 체세포핵이식, 경주개 동경이, 트립신, 일차배양

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